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Circadian modulation of motivation in mice

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Highlights

- Mice subjected to a 12:12-h light/dark cycle exhibited a diurnal rhythm in motivation for food reward, becoming more motivated during the night.

- The rhythm in motivation was also evident under constant dark conditions, denoting an endogenous circadian nature.
- The rhythm in motivation was also evidenced in aged mice, indicating that it was not affected by aging.
- Circadian arrhythmicity induced by chronic exposure to constant light conditions impaired motivation in mice, producing lower motivational levels.
- Day/night difference in motivation was also present in *ad libitum*-fed mice when using a palatable reward (chocolate).
- Total dopamine content within the Nucleus Accumbens did not present a day/night variation nor was affected by age.

Abstract

Most living organisms have a circadian timing system adapted to optimize the daily rhythm of exposure to the environment. This circadian system modulates several behavioral and physiological processes, including the response to natural and drug rewards. Food is the most potent natural reward across species. Food-seeking is known to be mediated by dopaminergic and serotonergic transmission in cortico-limbic pathways. In the present work, we show evidence of a circadian modulation of motivation for food reward in young (4-months old) and aged (over 1.5 years old) C57BL/6 mice. Motivation was assayed through the progressive ratio (PR) schedule. Mice under a 12:12 light/dark (LD) cycle exhibited a diurnal rhythm in motivation, becoming more motivated during the night, coincident with their active phase. This rhythm was also evident under constant dark conditions, indicating the endogenous nature of this modulation. However, circadian arrhythmicity induced by chronic exposure to constant light conditions impaired the performance in the task causing low motivation levels. Furthermore, the day/night difference in motivation was also evident even without caloric restriction when using a palatable reward. All these results were found to be unaffected by aging.

Taken together, our results indicate that motivation for food reward is regulated in a circadian manner, independent of the nutritional status and the nature of the reward, and that this rhythmic modulation is not affected by aging. These results may contribute to improve treatment related to psychiatric disorders or drugs of abuse, taking into account potential mechanisms of circadian modulation of motivational states.

Keywords: Circadian system, Motivation, Food reward, Dopamine, Nucleus Accumbens.

1. Introduction

Organisms exposed to daily environmental cycles display diurnal rhythms in physiology, metabolism and behavior. These rhythms are generated and sustained by cell-autonomous circadian clocks, which help organisms anticipate predictable changes in the environment. They continue to operate in constant environmental conditions (i.e., free-run) with a period of about 24 hours, indicating the endogenous nature of circadian rhythms (reviewed in [1]). In mammals, the master circadian oscillator is located in the suprachiasmatic nuclei (SCN) of the hypothalamus, and it is mainly synchronized by the light/dark (LD) cycle [2], which acts together with peripheral oscillators to keep daily and circadian rhythms. The synchrony or temporal coordination of circadian oscillators between central and peripheral tissues, and their alignment with the external environment, is extremely important for maintaining organism homeostasis [3].

The response to several types of reinforcers is modulated by the circadian system [4]. For example, pharmacological, physiological, and behavioral effects of reinforcing stimuli vary as a function of time of administration or availability over a 24-h cycle [5-7]. Mice carrying clock genes mutations display altered performance in a variety of reward-related behavioral tasks, such as drug seeking and sensitization

[8, 9]. Interestingly, most of the brain areas involved in reward processing, such as the ventral tegmental area (VTA) [10-12], the prefrontal cortex (PFC) [12], the amygdala (AMY) [13], and the nucleus accumbens (NAc) [12] express clock genes. Most of these areas are indeed peripheral circadian oscillators, suggesting a link between deregulation of circadian rhythms and psychotic disorders [14]. In addition, it was shown that components of the dopaminergic system - well known to be implicated in motivation and reward-related behaviors - were under circadian regulation. Circadian regulatory elements were found in the promoter regions of genes expressing monoamine oxidase A (MaoA) [11], tyrosine hidroxylase (TH) [15], dopamine transporter (DAT) [16], and dopamine receptor type 3 (DRD3) [17]. Furthermore, daily oscillations of DA total levels in the dorsal striatum were reported [18]. Overall, these data suggest that diurnal variations in dopaminergic metabolism and signaling could be in part responsible for rhythmicity in dopamine-mediated behaviors such as food seeking.

Food intake is regulated by complementary homeostatic and hedonic mechanisms. While hypothalamic nuclei mainly regulate the homeostatic drive of feeding, cortico-limbic structures control rewarded feeding behaviors [19, 20]. The Progressive Ratio (PR) schedule has been widely used to assay motivation for food reward. In this task, subjects must increase the number of responses made to earn subsequent rewards. The point at which a subject quits working for rewards is called the breaking point and serves as an index of motivation [21-23].

The aging process is known to involve neurochemical and neuroanatomical changes in the brain that ultimately leads to dysfunction of cognitive performance and loss of behavioral flexibility. Both dopaminergic and the serotonergic system are subject to change during aging [24], and many of the cognitive functions altered with advancing age require reward-based processing [24, 25]. On the other hand, it is also known that the circadian system is affected by aging. Age-related decline in circadian organization implies reduced amplitude and increased instability of circadian rhythms in many physiological and behavioral variables (reviewed in [26]).

The aim of this work is to present evidence on the diurnal and circadian modulation of motivation for food reward by using the PR schedule in mice. First, we tested

young adult mice in different lighting conditions and phases to address whether motivation is subjected to a circadian regulation. Then, we evaluated the motivation displayed by both young and aged mice in order to evidence whether an effect of aging on the circadian control on the motivated behavior exists. In addition, we measured total DA content in the NAc at different times of the day aiming to establish correlations with the behavioral findings.

2. Materials and Methods

2.1. Animals.

Mice (C57BL/6) were purchased from commercial suppliers (Faculty of Veterinary Sciences, University of Buenos Aires, Argentina) and were maintained in a 12:12-h light-dark cycle (LD, lights on at 0800 h) and room temperature set at 20 ± 2ºC with food and water *ad libitum* (except when noted). Young (4-month old) and aged (over 18 months of age) male mice were used throughout the experiments. When animals had to be handled in the dark, a dim red light source (< 5 lux) was used. The present experiments were approved by the Animal Care and Use Committee of the University of Quilmes (Buenos Aires, Argentina), and performed in strict accordance with NIH rules for animal care and maintenance.

2.2. Locomotor activity recording.

Animals were transferred to individual cages equipped with infrared sensors to detect locomotor activity, and with light intensity averaging 200 lux (fluorescent tube) at cage level. Total activity counts for each mouse were quantified as the total number of infrared sensor beam breaks and were stored at 5-min intervals for further analysis.

2.3. Experimental groups.

Motivation for food reward was assessed through the Progressive Ratio (PR) task in young and aged mice under different experimental conditions: 12:12-h light/dark (LD) cycle, constant dark (DD) and constant light (LL). Different cohorts of mice were tested in each condition in order to minimize the effects of multiple exposure to the

PR task and therefore avoid habit formation [27]. Firstly, young mice under a 12:12 LD cycle were evaluated at different time points or Zeitgeber times (ZTs) within the light phase (at ZT 2, ZT 4 and ZT 6) or the dark phase (at ZT 14, ZT 16 and ZT 18). By convention, ZT 12 is defined as the beginning of the dark phase. Then, in order to minimize the number of animals used and because no differences in mice performance were found between ZTs belonging to the same phase of the cycle (i.e., within the day or the night, see Figure 1), the following experiments evaluated the PR task at only one time point per cycle phase. Therefore, when comparisons between groups are shown, the performance at the middle of the day (ZT 6) or at the middle of the night (ZT 18) for LD groups is taken into account (Supplementary Figure 1A). Aged animals under a LD cycle were only evaluated at either ZT 6 or ZT 18.

Secondly, for constant dark (DD) experiments, animals were kept under constant darkness for at least 7 days before the start of the behavioral experiments, and were evaluated either in the middle of their subjective day at Circadian time (CT) 6 or in the middle of their subjective night at CT 18 (Supplementary Figure 1B). By convention, CT 12 is defined as the onset of locomotor activity.

Finally, for constant light (LL) experiments, animals were continuously exposed to light (200 lux at cage level, fluorescent tube) for at least 25 days before the start of the behavioral experiments to induce circadian arrhythmicity (confirmed by locomotor activity recordings). Arrhythmic mice were tested at the same clock hours as their control littermates in LD conditions (Supplementary Figure 1C). Since there were no time cues under LL - therefore, no phase or ZT could be estimated - and no significant differences were found between the evaluation time points, results from the LL group were pooled and are shown as one data set independently of evaluation time. Representative actograms of total locomotor activity are depicted in Supplementary Figure 2. Lack of circadian rhythmicity under LL conditions was confirmed by Lomb-Scargle periodograms by taking the previous 7 days to the start of behavioral experiments.

In all the experimental groups mentioned above, mice were subjected to caloric restriction – by controlling the daily amount of food received – starting 7 days prior

to the experiment, in order to keep them at 85-90% of their free-feeding weight. Under these conditions, mice were willing to work (press the lever) to obtain a regular food pellet as a reward while performing the task. In all cases, food was provided immediately after sessions. On the other hand, additional groups of mice were tested for their motivation to obtain a palatable reward (20 mg chocolate flavored pellets) without being previously subjected to caloric restriction. In these cases, young and aged mice under LD conditions had *ad libitum* food access in their home cages throughout the experiment and were tested in the PR task using chocolate pellets as a reward at ZT 6 and ZT 18.

2.4. Apparatus.

The experimental apparatus consisted of 4 matching lever boxes (Model ENV-307A, Med Associates, St. Albans, VT) housed in sound-attenuating chambers (Model ENV-021M; Med Associates). The dimensions of each lever box were 21.59 x 17.78 x 12.70 cm. The ceiling, side walls, and door of each box were made from clear Plexiglas. The front and back walls were stainless-steel panels and the floor was made of parallel stainless-steel bars. The front wall of each box contained left and right retractable levers; a food cup was located between the levers and a cue light was located directly above the food cup. A pellet dispenser delivered food reward into the food cup. Reward consisted of either 20-mg grain-based food pellets (Bio-Serv, Frenchtown, NJ) or 20-mg chocolate pellets, depending on the experiment. The back wall of each box contained a house light (14-W, 100 mA) directed towards the ceiling. The operant chambers were controlled by the Med-PC IV software package. The fan was ON throughout the session. A PC attached to an electronic interface (MED Associates, Inc., Model DIG-700 and SG-215) was used to control the experimental equipment and record the data. The time of each lever press was recorded to an accuracy of 10 ms and placed into 1-s time bins.

2.5. Motivation for food reward.

Progressive ratio (PR) training was used to assess the effort a mouse was willing to expend to get a reward, in this case a food pellet [21]. Mice were evaluated in two

consecutive phases: 1) operant lever press training, and 2) progressive ratio (PR) schedule. In all cases, the animals were weighed before each session.

1) *Operant lever press training*. All mice were given 1 daily session of lever-press training for three consecutive days. One lever – left or right, balanced among subjects – was presented during the session. Each lever press resulted in the delivery of a food pellet. Sessions ended after the mouse received 60 food pellets or 60 min had passed, whichever came first.

2) *Progressive ratio (PR) schedule.* After operant lever press training, mice received only one session of PR training. Briefly, one lever was extended at the beginning of the session, and the reward was delivered only after the mouse has completed a certain number of lever presses. The number of lever presses needed to obtain the reward in each trial within a session was derived from the following equation [21]:

$$P = \left[5 \times e^{(i \times 0.2)}\right] - 5$$

where *P* is the required number of lever presses (rounded to the nearest integer) and *i* refers to the trial number. This equation results in the following arithmetic series: 1, 2, 4, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145.178, 219, 268, 328, 402, 492, 603, 737, 901, 1102, 1347, 1647, 2012, etc. Therefore, the threshold was set at one lever press to obtain the food reward in the first trial, two lever presses for the second trial, four lever presses for the third trial, and so forth. The session ended after 2 h or after 10 min had elapsed without a lever press. The “breaking point” was defined as the number of lever presses the animal had to complete in a trial in order to get a reward but was unable to achieve. Motivation was measured by recording the total number of lever presses performed along the session, the total number of rewards earned, the breaking point, and the percent of subjects that continued performing the task as a function of session duration (survival %).

2.6. Dopamine determination.

Mice were anesthetized with isoflurane and euthanized by cervical dislocation at either the middle of the day (ZT 4-8) or the middle of the night (ZT 16-20). The brains were quickly removed and frozen at -80 °C. Samples from ventral striatum from each hemisphere were taken using a micro puncher. Tissue was homogenized in 1 ml of

0.3 M perchloric acid, centrifuged for 15 min at 3000 g at 4 °C and then frozen at -80 °C. Dopamine levels were measured by high pressure liquid chromatography coupled to electrochemical detection (HPLC-EC) using a Phenomenex Luna 5 µm, C18, 250 mm × 4.60 mm column (Phenomenex, Torrance, CA, USA) and LC-4C electrochemical detector with glassy carbon electrode (BAS). The working electrode was set at +0.65 V versus an Ag/AgCl reference electrode. The mobile phase contained 0.76 M NaH2PO4·H2O, 0.5 mM EDTA, 1.2 mM 1-octane sulfonic acid, and 5% acetonitrile; pH was adjusted to 3.0. The variation coefficient of the technique was less than 5% and the lower limit of detection of MD was 5.0 ng/ml. Intra-day and inter-day coefficient of variation was 3.2 and 13.2%, respectively. Dopamine quantification was referred to total protein content. Proteins were measured by using the NanoDrop 1000 Spectrophotometer (Thermo Scientific).

2.7. Data analysis.

Lomb-Scargle periodograms were performed to assess circadian rhythmicity by using Actogram J software (NIH, Bethesda, MD, USA). For the experiment in which motivation in young mice was assessed at different time points throughout the day, the results (i.e., number of lever presses, the number of rewards earned and breaking point) were analyzed by one-way analysis of variance (ANOVA) followed by post-hoc comparisons. When equality of variances was not met, Welch's correction was applied.

For the experiments in which the parameters Light Conditions [LD, DD], Age [Young, Aged] and Phases [Day, Night] were compared, a linear model was fit to the data obtained from the variables measured (Lever presses, Rewards earned and Breaking Point measurements) using the package "*nlme"* in R [28, 29]. A logarithmic scale was applied to Lever Presses and Breaking Point data sets in order to improve the normality of the data. The model was built evaluating every variable as a function of Age interacting with Phase and Light Conditions. Then, a Wald F test was fit for each model analyzing the main effects, double and triple interactions. Post-hoc multiple comparisons were run between selected pairs of variables (Day vs Night in LD and Day vs Night in DD for young mice, Day vs Night in LD and Day vs Night in

DD for aged mice) using the Tukey's test contained in the package "*multcomp"* in R [30]. When significant interactions occurred between parameters of interest, a simple effects linear model was applied in order to address the source of the interaction. In those cases, Bonferroni's corrections were applied to the p values obtained. Effect sizes were calculated using the Eta Squared function contained in the package *"lsr"* in R [31]. The values obtained were compared to a table from [32] to interpret the size of the effect.

Results from mice under LL conditions were analyzed by one-way ANOVA followed by post-hoc comparisons. Data obtained from the experiments in which mice were tested without caloric restriction were evaluated using a two-tailed t-test. When equality of variances was not met, Welch's corrections were applied.

In all cases, performance in the progressive ratio schedule was evaluated through the Kaplan-Meier survival function [22]. A log-rank (Mantel-Cox) test was used to determine survival differences between groups and Bonferroni's corrections were applied to p values for multiple comparisons.

Dopamine content was analyzed by two-way ANOVA.

Statistical analyses were performed using Graphpad Prism (GraphPad Software Inc., CA, USA), IBM SPSS Statistics for Windows (IBM Corp., Armonk, NY, USA) and R [28]. In all cases, the alpha level was set at $p<0.05$.

3. Results

3.1. Young mice display a diurnal rhythm in motivation.

Motivation in calorie-restricted young male mice under a 12:12-h LD cycle was evaluated using the progressive ratio (PR) schedule of reinforcement at different time points during the day (ZT 2, ZT 4 and ZT 6) and during the night (ZT 14, ZT 16 and ZT 18). In this test, mice are required to make an increasing number of operant responses in order to get every successive reward. Examination of the total number of lever presses made along the session revealed that the groups tested at night presented higher values compared to the groups tested during the daytime ($p<0.0001$, one-way ANOVA with Welch's correction, Figure 1A). The total number of rewards earned, and the breaking point also displayed significant higher values

for the night groups (Rewards: $p<0.0001$, one-way ANOVA, Figure 1B; Breaking point: $p<0.0001$, one-way ANOVA with Welch's correction, Figure 1C). *Post-hoc* comparison revealed no significant differences between the time points (ZTs) evaluated within the day or the night (see figure caption in Figure 1). Additionally, survival percentages from the ZTs corresponding to the night phase had significant differences compared to those corresponding to the day phase, being higher for the groups evaluated during the night ($p<0.0001$ for ZT14 vs ZT2, ZT14 vs ZT4, ZT14 vs ZT6, ZT16 vs ZT2, ZT16 vs ZT4 and, ZT16 vs ZT6; $p=0.0315$ for ZT18 vs ZT2; $p=0.003$ for ZT18 vs ZT4; $p=0.027$ for ZT18 vs ZT6; Mantel-Cox test, Figure 1D). Taken together, these results indicate a strong diurnal variation in motivation in young mice, with the highest motivation rates exhibited during the night.

Given that no significant differences were observed for the time points within the day or within the night, the following experiments were performed in two time points representing the mid-day phase or the mid-night phase (see Materials and Methods).

---------- Insert Figure 1 about here ----------

3.2. The daily rhythm in motivation persists in constant darkness, is not affected by aging and is modulated by light conditions.

Young (4 months) and aged mice (18 months) were subjected to constant darkness conditions (DD) or a 12:12 light/dark (LD) cycle. They were tested using the PR schedule during the middle of the day (ZT 6) or night (ZT 18) in LD, and in the middle of their subjective day (CT 6) or their subjective night (CT 18) in DD. The results from the variables measured were fit to a linear model to study the interactions between the parameters studied.

As previously shown, young mice display a daily rhythm in motivation, with highest values during the night ($p<0.001$ for Lever presses, Rewards, and Breaking Point; Tukey's test). We also found that this rhythm persisted in constant darkness conditions, denoting its endogenous nature. The total number of lever presses ($p=0.006$, Tukey's test, Figure 2A), rewards earned ($p=0.002$, Tukey's test, Figure 2B) and breaking point ($p=0.004$, Tukey's test, Figure 2C) showed higher values

during the subjective night compared to the subjective day. Survival curves also presented higher percentages for the night compared to the day in mice under LD cycle, and for the subjective night compared to subjective day in mice under DD ($p<0.0001$ for LD night vs day and DD subj. night vs subj. day; Mantel-Cox test, Figure 2D).

---------- Insert Figure 2 about here ----------

These rhythms in motivation were also present in aged mice. Motivation was higher during the night in LD and during the subjective night in DD conditions, measured by total lever presses ($p<0.001$ for LD, $p=0.002$ for DD, Tukey's test, Figure 3A), number of rewards obtained ($p<0.001$ for LD, $p=0.001$ for DD, Tukey's test, Figure 3B), and breaking point ($p<0.001$ for LD and $p=0.002$ for DD, Tukey's test, Figure 3C). In line with the previous results, the survival functions obtained from aged mice evaluated under both LD and DD conditions showed significant differences between day and night, with higher values during the night or subjective night ($p<0.0001$ for LD night vs day and, $p=0.048$ for DD subj. night vs subj. day; Mantel-Cox test, Figure 3D).

---------- Insert Figure 3 about here ----------

The linear model also revealed that the circadian variation is not affected by aging (LM, Phase factor $p<0.001$ for Lever Presses, Rewards and Breaking Point, Age factor $p=0.140$ for Lever presses; $p=0.116$ for Rewards, and $p=0.106$ for Breaking Point). The interaction between Age and Phase factors was not significant for any of the behavioral outputs measured (see Supplementary Table 1).

The light conditions to which mice have been exposed before and during the testing (an alternating 12:12 LD cycle or constant darkness) were found to significantly affect the performance in the PR task for all the outputs measured. Thus, mice subjected to DD showed higher motivation levels when compared to the under a LD

cycle (LM, Light condition factor $p<0.001$ for Lever Presses, Rewards, and Breaking point).

The model revealed some significant double interactions involving the factor Light Conditions. The interaction between Light Conditions and Phase was significant for the variables Lever Presses and Breaking Point (LM, Phase: Light Condition factor: $p=0.029$ for Lever presses, $p=0.087$ for Rewards, and $p=0.046$ for Breaking Point) suggesting that the environmental lighting conditions to which animals are exposed exert a differential effect on motivation depending on the phase (Day or Night) in which this behavior was assessed.

We were particularly interested in this last interaction, given that a previous study suggested that light can exert an acute effect on mood and behavior bypassing the circadian clock [33]. In order to address the source of the interaction between the Light Condition and Phase, a simple-effects linear model was applied for each of the evaluated phases (Day and Night). The models showed that the LD cycle impaired the motivation displayed by the mice in both phases. However, the motivation displayed during the day was the most dramatically affected as indicated by the effect sizes obtained for all the outputs measured (LM, Light Conditions factor for Lever Presses: Phase=Day: $p>0.001$, etaSq=0.266; Phase=Night: $p=0.026$, etaSq=0.073; for Rewards: Phase=Day: $p>0.001$, etaSq=0.271; Phase=Night: $p=0.034$, etaSq=0.067; for Breaking Point: Phase=Day: $p>0.001$, etaSq=0.271; Phase=Night: $p=0.028$, etaSq=0.071). These results indicate that motivation levels are affected in mice subjected to an alternating LD cycle compared to mice in DD. In addition to this global effect of the LD cycle on motivation, light during the day appears to have an acute effect on the behavior studied.

3.3. Circadian arrhythmicity affects motivation.

Constant light exposure is known to cause period lengthening followed by arrhythmicity in mice [34]. With the objective to explore the effect of circadian arrhythmicity on motivation, young and aged mice previously subjected to constant light conditions (LL) for several weeks were evaluated on the PR task. It is not possible to test arrhythmic mice at a certain time, because there is no external cue

or ‘internal time’ to define a time point or phase for them. For this reason, arrhythmic mice were tested at different clock hours and results were pooled since no significant differences between time points were found (data not shown). Since previous results from our laboratory evidenced that constant light exposure impaired cognitive function in mice [35], we expected that constant light exposure would affect motivation. Therefore, performance of mice under LL was compared with the groups that presented the lowest and highest motivation levels (i.e., the LD day and night groups, respectively).

Results obtained from young mice evidenced that the LL group presented a decreased performance in the PR task compared to the group evaluated during the night, but similar performance when compared to the group evaluated during the day, as seen for the total number of lever presses ($p<0.0001$, one-way ANOVA with Welch’s correction. $p=0.001$ for LL vs LD night and $p=0.48$ for LL vs LD day, Games-Howell posttest, Figure 4A), rewards earned ($p<0.0001$, one-way ANOVA. $p<0.0001$ for LL vs LD night and $p=0.744$ for LL vs LD day, Tukey’s multiple comparison test, Figure 4B), and breaking point ($p<0.0001$, one-way ANOVA with Welch’s correction. $p=0.001$ for LL vs LD night and $p=0.574$ for LL vs LD day, Games-Howell posttest, Figure 4C). Interestingly, mice under LL displayed significant differences in survival curves with the LD day group but similar survival percentages with the LD night group ($p<0.0001$ for LL vs LD day, $p=0.0669$ for LL vs. LD night; Mantel-Cox test, Figure 4D).

Aged mice under chronic constant light conditions evidenced an ‘intermediate’ motivation when compared to their control littermates under a regular LD cycle tested during the day and the night. In this sense, the LL group presented significant differences with both LD day and night groups for total lever presses ($p<0.0001$, one-way ANOVA with Welch’s correction. $p=0.011$ for LL vs LD night and $p=0.002$ for LL vs LD day, Games-Howell posttest, Figure 5A), rewards earned ($p<0.0001$, one-way ANOVA. $p=0.018$ for LL vs LD night and $p=0.009$ for LL vs LD day, Bonferroni’s multiple comparison test, Figure 5B), and breaking point ($p<0.0001$, one-way ANOVA with Welch’s correction. $p=0.011$ for LL vs LD night and $p=0.003$ for LL vs LD day, Figure 5C). In addition, survival curves of aged mice under LL conditions

displayed significant differences only with the LD day group ($p<0.0001$ for LL vs LD day, $p=0.2202$ for LL vs LD night; Mantel-Cox test, Figure 5D).

In summary, these results suggest that circadian arrhythmicity has a negative effect on motivation, with a stronger outcome in young mice.

---------- Insert Figure 4 and 5 about here ----------

3.4. The daily variation in motivation persists in *ad libitum*-fed mice.

To verify that the daily variation in motivation performance observed in mice was not an effect induced by the caloric restriction applied, the PR schedule was used to evaluate motivation in *ad libitum*-fed mice. Both young and aged mice were subjected to a 12:12-h LD cycle and tested during the day or night. Because free-fed mice displayed no interest on regular pellets as a reward (data not shown), palatable chocolate pellets were used instead of regular food pellets.

For both young and aged mice, motivation was higher during the night compared to the day, as observed for the total number of lever presses ($p=0.0191$ for young, $p=0.0004$ for aged; two-tailed t-test without and with Welch's correction, respectively, Figures 6A and 7A), rewards earned ($p=0.0077$ for young, $p<0.0001$ for aged; two-tailed t-test, Figures 6B and 7B), and breaking point ($p=0.0221$ for young, $p=0.0003$ for aged; two-tailed t-test without and with Welch's correction, respectively, Figures 6C and 7C). Additionally, survival curves also presented higher percentage values for the night groups ($p=0.005$ for young, $p<0.0001$ for aged; Mantel-Cox test, Figures 6D and 7D).

These results corroborate the day/night differences previously observed. On the other hand, in mice without nutrient imbalance, the use of chocolate pellets as a reward activates different (hedonic) components of motivation pathways. Accordingly, the rhythm in motivation is not only influenced by nutritional deficits, but the diurnal variation also persists when palatable food is used as a reinforcer and, therefore, other aspects of motivation are being assessed.

---------- Insert Figures 6 and 7 about here ----------

3.5. Total dopamine content in the Nucleus Accumbens does not display a diurnal variation.

The reward system is mediated by the mesolimbic dopaminergic pathways within the corticolimbic areas. In addition, diurnal rhythms in several components of dopaminergic signaling and in clock core proteins within the Nucleus Accumbens (NAc) have been found [36, 37]. Following this line of evidence, we wanted to explore whether the daily variation in motivation could be correlated to differences in striatal dopamine (DA) content present in the NAc. Supplementary Figure 3 shows total DA content in NAc measured during the same time of day that the behavioral task performed under the LD cycle (middle of the day and middle of the night) for both young and aged mice. There were no significant differences in age or time of day factors for DA content ($p=0.3744$ for Time of day, $p=0.50$ for Age; two-way ANOVA). These results indicate that the observed day/night differences in behavior are not the direct consequence of a different DA content in the NAc, but also that total dopamine levels do not decline with aging within the NAc.

4. Discussion

In the present study, robust variations in motivation for food reward were observed in young and aged mice. This rhythm was also sustained in constant darkness conditions (DD), suggesting that this variation in motivation is endogenous and constitutes a circadian rhythm. Mice exhibited higher motivation for food reward during the nighttime (their active phase) compared to the daytime (their resting phase). Under constant darkness conditions, motivation was higher during the subjective night. These results are in accordance with previous reports of daily rhythms in other motivated behaviors such as drug-seeking and consumption, and sex-related rewards [7, 38]. The results showed in the present work incorporate novel information concerning the circadian modulation of reward-related processes involving a natural reinforcer.

Motivation and reward-related behaviors are thought to be comprised of different components, such as the “liking” or hedonic component and the “wanting” or

incentive salience component [39]. In most of our experiments, subjects were motivated to work for a food reward due to their physiological nutrient imbalance. That is, mice were calorie-restricted and maintained on 85-90% of their *ad libitum*-feeding weight, which increased their motivation for the appetitive reward. Despite their physiological deficit, animals displayed a clear day/night difference in motivation, with a higher nocturnal response. Our results are in accordance with the foraging role of the circadian system and highlight the importance of an adequate modulation of motivational behavior in order to encourage the search for food and survival.

The linear model applied to our data revealed a profound effect of lighting conditions (i.e., LD vs. DD) on motivation. We found that the LD conditions cause a generalized decrease in motivation in both phases tested (Day and Night), as compared to the DD conditions. Most importantly, we demonstrated that the effect of the alternating light/dark cycle exerted a more dramatic consequence on the motivated behavior displayed during the day. These results are in line with previous studies that indicate that light affects mood and cognition acting on a retina-brain SCN-independent pathway [33, 40]. We hypothesize that even though the daily rhythm in motivation found in the present study is modulated by the circadian clock, it may be also affected by light in an acute way. In this sense, both the endogenous circadian clock and the lighting conditions may have a synergistic effect on motivation. Although some reports interpret DD conditions as depressogenic in mice and rats - based on forced swim test, sucrose consumption, etc. [41, 42] - it is not clear whether these symptoms would be associated with reduced motivation. Moreover, these studies use long-term light deprivation (4 to 6 weeks in DD) as a paradigm for depression-like behavior, while in the present study animals are kept in DD for 7 days before the start of the behavioral experiments.

Furthermore, the acute effect of light mentioned above might also be playing a role in decreasing the motivation in mice subjected to LL conditions. Besides the limitation of this experiment, in which it was not possible to define different time points for mice evaluated in this condition because of the lack of a time reference, all animals exhibit similar low motivation levels. This effect of decreased motivation

is mainly motivational/rewarding rather than motor, since in the operant conditioning phase there were no significant differences between groups (data not shown). These results complement previous studies from our group indicating that circadian arrhythmicity induces loss of temporal control in an interval timing task in mice involving food reward [18, 35] (but see [43]). The low motivation found in mice under LL conditions was comparable to the levels displayed by the mice tested during the light phase of the LD cycle for young mice and, for aged mice, intermediate between diurnal and nocturnal levels. It is hard to dissect the effects of circadian arrhythmicity and the effect of the light *per se*; however, the homogeneously low motivation levels displayed by animals under LL suggests that light is pushing motivation levels down and making them less disperse compared to mice tested during the night. On the other hand, for both young and aged mice under LL conditions, survival percentages were similar to the LD night group but different - and also higher - than the LD day group. Nonetheless, further experiments will be needed to clearly dissociate the effects of light and circadian arrhythmicity on motivation.

In order to evaluate if hedonic components of motivation could also present a daily variation in the PR task, mice with satisfied nutritional requirements have been evaluated by using a palatable food reward. Our results show that the daily and circadian variation was maintained in mice without calorie restriction when chocolate was used as a reward. A previous work [44] shows concordant results in C56BL/6 mice in a place preference sucrose test, in which a daily rhythm in sucrose intake with greater consumption during the dark phase was found. However, this rhythm was impaired in the arrhythmic circadian mutants *Per2*^{Brdm1} and double *Per1*$^{-/-}$ *Per2*^{Brdm1} mice even when tested in LD [45]. Overall, our results suggest that the circadian system may influence different aspects of motivated and reward-related behaviors, including both the physiological driven states - that promote food consumption - and the hedonic aspects associated with feeding.

It is well known that aging is a process that involves physiological changes in the brain that account for behavioral changes. While several studies have found that aging involves general cognitive decline, and changes in robustness and stability of circadian rhythms, the effect of this process on hedonic behaviors is not fully

understood [46-48]. In the present work we have demonstrated that the performance in the PR schedule is not affected by aging, but more importantly, we found that the rhythm in motivation is preserved in aged mice. These results are in accordance with a previous study that suggested that motivation is not affected by aging when mice were evaluated in an operant conditioning test using sweetened sugar as a reward [49]. In addition, reward processing has long been associated with dopaminergic signaling in the ventral striatum [50-52]. In the present study, we found no evidence of aging or time of day affecting total DA levels in the Nucleus Accumbens. A possible limitation in this experiment, however, was the method applied for measuring DA in the NAc. By measuring total DA content, our results do not account for possible daily variations in DA storage inside the synaptic vesicles and/or differences in DA levels released to the extracellular space. More appropriate techniques, such as microdialysis [53], could be used for future work in order to corroborate these assumptions and to establish correlations with our behavioral results. On the other hand, in agreement with our findings, recent reports have indicated that some aspects of reinforcement, such as hedonia, do not appear to be strictly DA-dependent [54]. In this regard, future work should be focused on the role of dopaminergic signaling in motivated behaviors by studying the circadian modulation of specific receptors or signaling pathways, as well as to explore other systems associated to reward-processing, such as the serotonergic or the endogenous opioid system.

Motivated behaviors are particularly relevant in human disease. In addition, there is a role for the circadian clock in the regulation of human reward motivation and substance abuse and dependence [9, 10, 55-59]. For these reasons, we believe that our findings contribute to the understanding of the importance of differential (and time-dependent) treatment of psychiatric disorders, addictions, and motivational deficits.

5. Conclusions.

To our knowledge, this is the first report of a circadian effect in motivation in mice by using the progressive ratio task and a natural reinforcer. Indeed, it is also the first

report indicating that the circadian effect is maintained in aged animals, pointing to the importance of the circadian system throughout lifetime. Our results also indicate a note of caution when interpreting behavioral results of experiments performed under a single time-point. In a broader context, our findings suggest that the circadian modulation of motivation is a robust feature, highlighting the importance of the interaction between the circadian and reward systems.

Authors declare no conflict of interest.

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Figure captions.

Figure 1. Daily rhythm in motivation for food reward in young mice. Animals under a 12:12 light/dark (LD) cycle were evaluated using the progressive ratio (PR) task at ZT 2, 4, 6 (daytime) and ZT 14, 16, 18 (nighttime). (A) Total number of lever presses ($p<0.0001$, one-way ANOVA with Welch's correction), (B) total number of rewards earned ($p<0.0001$, one-way ANOVA), (C) breaking point ($p<0.0001$, one-way ANOVA with Welch's correction), and (D) survival functions for session duration ($p<0.0001$ for ZT14 vs ZT2,4 and 6; $p<0.0001$ for ZT16 vs ZT2,4 and 6; $p=0.0315$ for ZT18 vs ZT2; $p=0.003$ for ZT18 vs ZT4; and $p=0.027$ for ZT18 vs ZT6; Mantel-Cox test). Data are expressed as mean ± S.E.M (n=9-12 per data point). *Post-hoc* comparison for lever presses: *$p<0.05$ for ZT14 vs ZT2 and ZT4, for ZT16 vs ZT2, ZT4 and ZT6, and for ZT18 vs ZT4. For rewards: *$p<0.05$ for ZT14 vs ZT6, and for ZT16 vs ZT6; **$p<0.01$ for ZT18 vs ZT6; ***$p<0.001$ for ZT14 vs ZT2 and ZT4, for ZT16 vs ZT2 and ZT4, and for ZT18 vs ZT2 and ZT4. For breaking point: *$p<0.05$ for ZT14 vs ZT2 and ZT4, for ZT16 vs ZT2 and ZT4, and for ZT18 vs ZT2 and ZT4. Tukey's Multiple Comparison test or Games-Howell post-hoc comparison test.

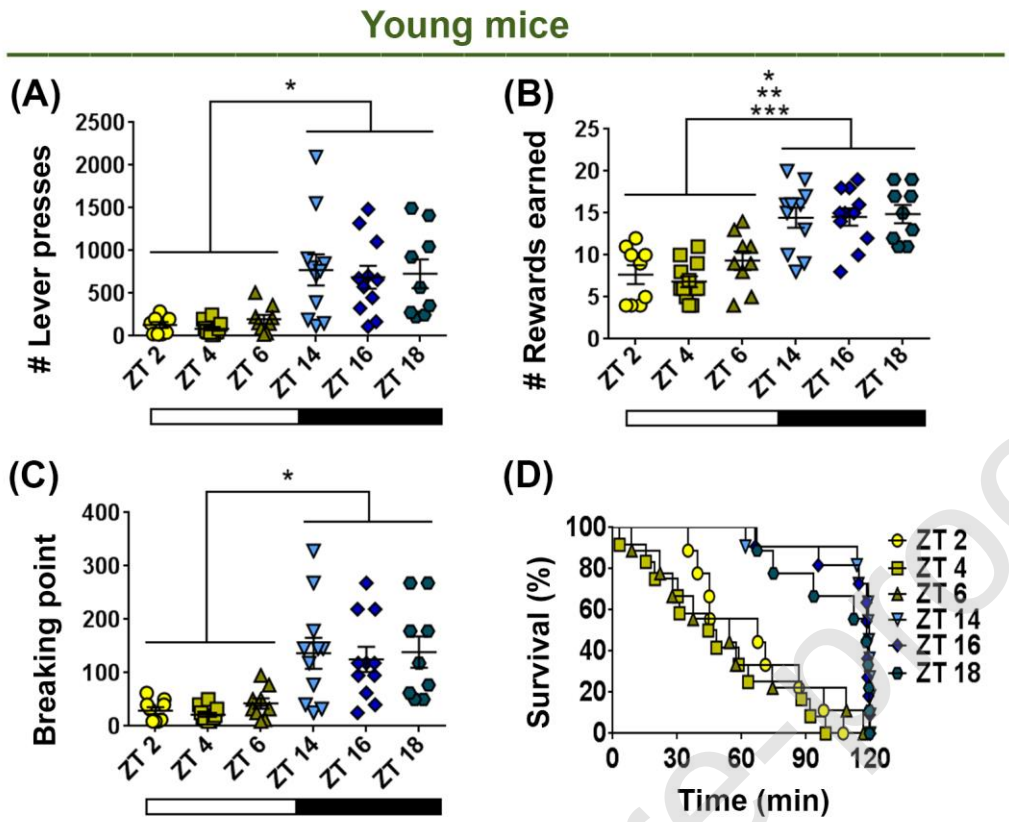

Figure 2. Day/Night rhythm in motivation persisted in DD conditions in young mice. Mice were evaluated in the middle of the day (LD day) or night (LD night) for LD condition, and in the middle of their subjective day (DD s. day) or subjective night (DD s. night) for DD condition. (A) Total number of lever presses ($p<0.001$ LD day vs LD night, $p=0.006$ DD s. day vs DD s. night, Tukey's test), (B) total number of rewards earned ($p<0.001$ LD day vs LD night, $p=0.002$ DD s. day vs DD s. night, Tukey's test), (C) breaking point ($p<0.001$ LD day vs LD night, $p=0.004$ DD s. day vs DD s. night, Tukey's test), and (D) survival functions for session duration ($p<0.0001$ LD day vs LD night, $p<0.0001$ DD s. day vs DD s. night, Mantel-Cox test). Data are expressed as mean ± S.E.M (n=18 for LD day and LD night, n=15 for DD s. night, and n=13 for DD s. day). **p<0.01, ***p<0.001, linear model followed by Tukey's test for *post-hoc* multiple comparisons. See Supplementary Table 1 for full statistics from the linear model.

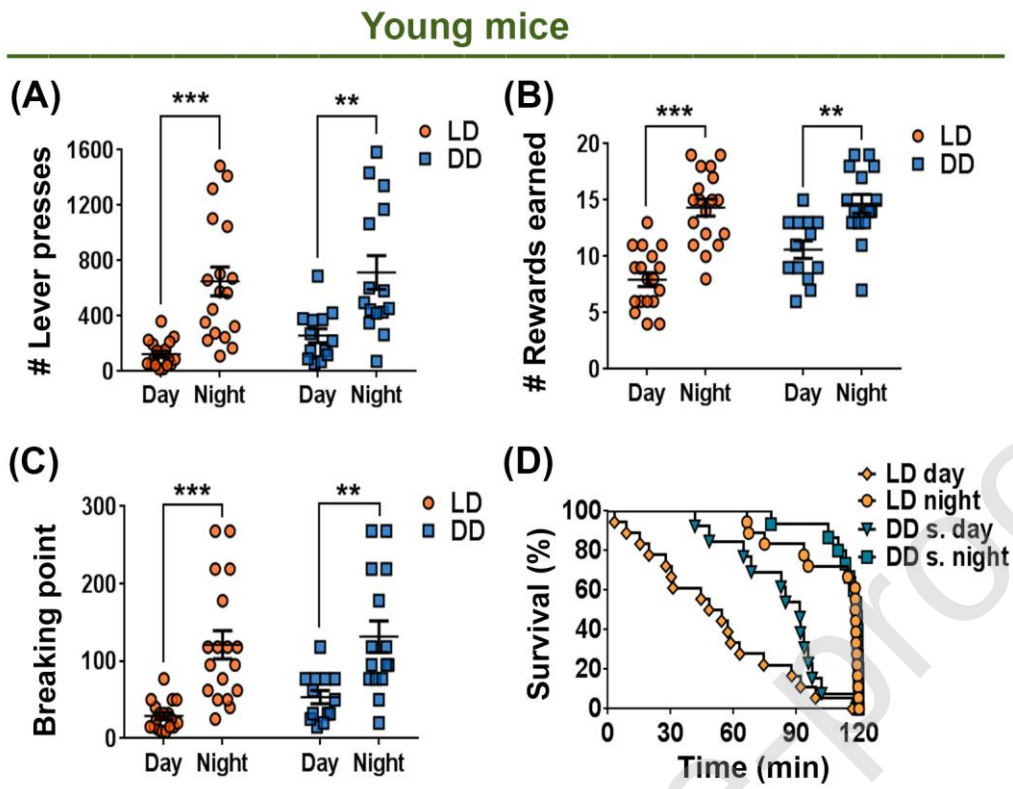

Figure 3. Rhythms in motivation in aged mice subjected to LD and DD conditions. Mice under LD or DD were tested during the middle of the day (LD day or DD s. day, respectively) or the middle of the night (LD night or DD s. night, respectively). (A) Total number of lever presses ($p<0.001$ LD day vs LD night, $p=0.002$ DD s. day vs DD s. night, Tukey's test), (B) total number of rewards earned ($p<0.001$ LD day vs LD night, $p=0.001$ DD s. day vs DD s. night, Tukey's test), (C) breaking point ($p<0.001$ LD day vs LD night, $p=0.002$ DD s. day vs DD s. night, Tukey's test), and (D) survival functions for session duration ($p<0.0001$ LD day vs LD night, $p=0.048$ for DD s. day vs DD s. night, Mantel-Cox test). Data are expressed as mean ± S.E.M ($n=13$ for LD day, $n=23$ for LD night, $n=15$ for DD s. day and DD s. night). **$p<0.01$, ***$p<0.001$, linear model followed by Tukey's test for *post-hoc* multiple comparisons. See Supplementary Table 1 for full statistics from the linear model.

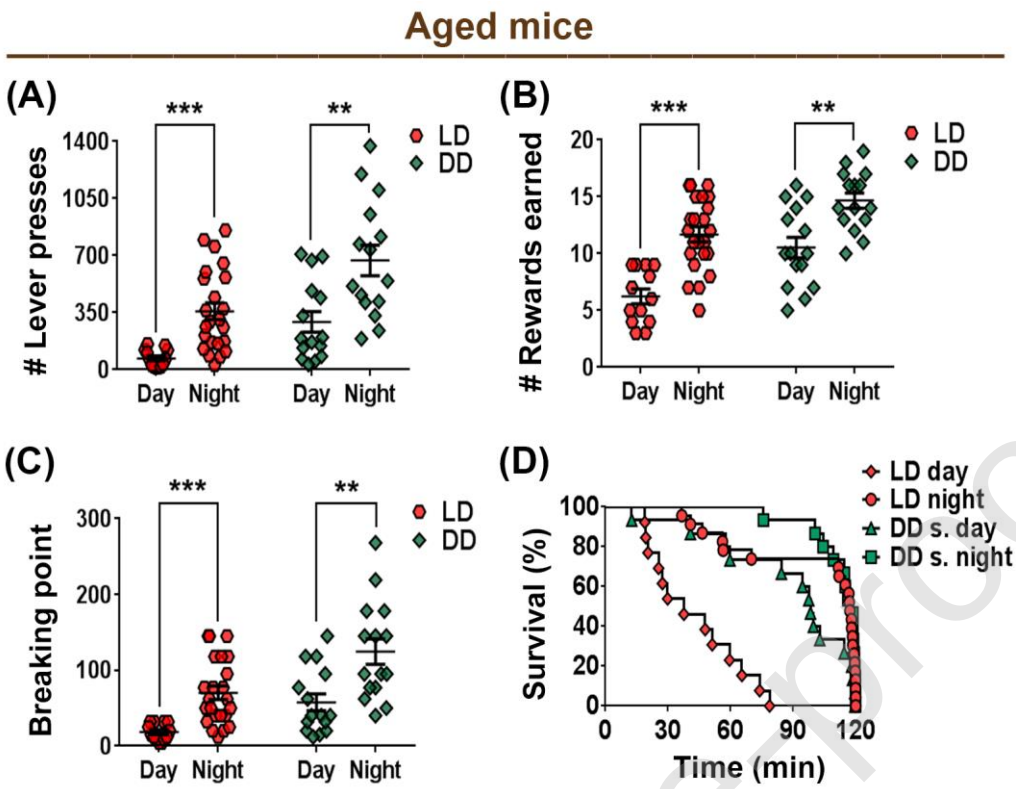

Figure 4. Motivation for food reward in constant light conditions in young mice. Animals were kept under constant light (LL) until circadian arrhythmicity in locomotor activity was evidenced and evaluated at the same clock hours as their controls under LD conditions. Results from the LL group are pooled and showed as one data set. (A) Total number of lever presses ($p<0.0001$, one-way ANOVA with Welch's correction), (B) total number of rewards earned ($p<0.0001$, one-way ANOVA), (C) breaking point ($p<0.0001$, one-way ANOVA with Welch's correction), and (D) survival functions for session duration ($p<0.0001$ for LL vs LD day, $p=0.0669$ for LL vs LD night, Mantel-Cox test). Data are expressed as mean ± S.E.M ($n=23$ for LL, $n=18$ for LD day and night). *Post-hoc* comparisons for lever presses: $p=0.001$ for LL vs LD night, $p=0.48$ for LL vs LD day. For rewards: $p<0.0001$ for LL vs LD night, $p=0.744$ for LL vs LD day. For breaking point: $p=0.001$ for LL vs LD night, $p=0.574$ for LL vs LD day. ***$p<0.001$, ****$p<0.0001$, Games-Howell posttest or Tukey's multiple comparison test.

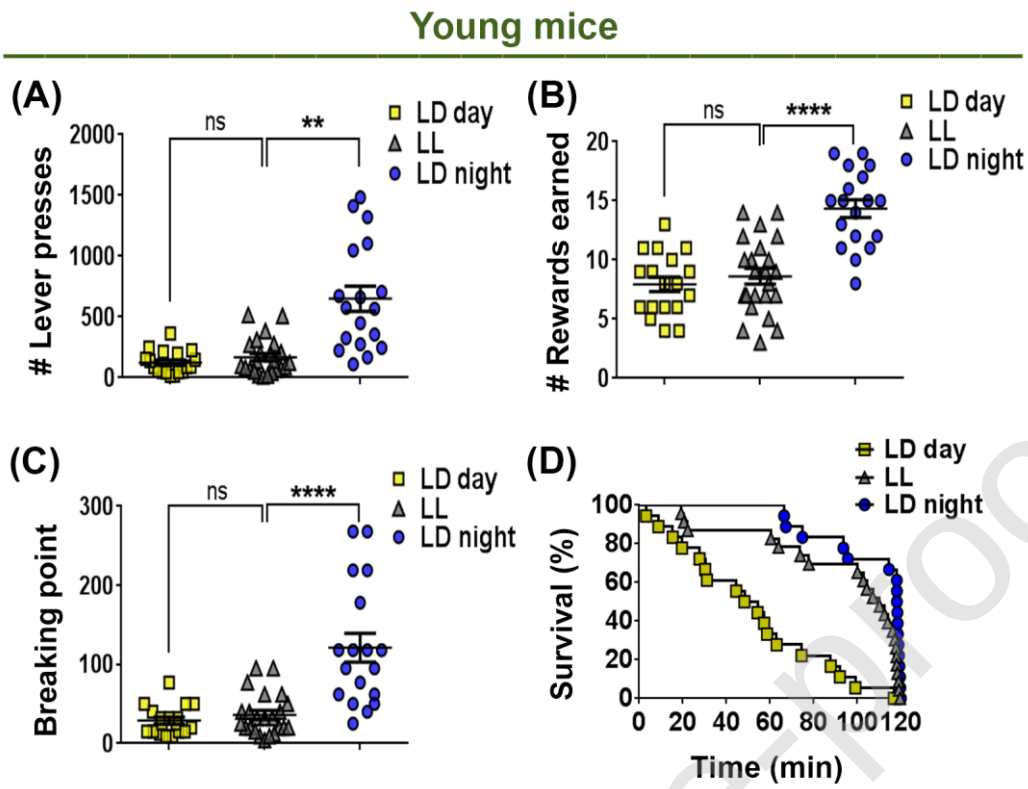

Figure 5. Motivation for food reward in constant light conditions in aged mice. Animals were kept under constant light (LL) until circadian arrhythmicity in locomotor activity was evidenced and evaluated at the same clock hours as their controls under LD conditions. Results from the LL group are pooled together and showed as one data set. (A) Total number of lever presses ($p<0.0001$, one-way ANOVA with Welch's correction), (B) total number of rewards earned ($p<0.0001$, one-way ANOVA), (C) breaking point ($p<0.0001$, one-way ANOVA with Welch's correction), and (D) survival functions for session duration ($p<0.0001$ for LL vs LD day, $p=0.2202$ for LL vs LD night, Mantel-Cox test). Data are expressed as mean ± S.E.M ($n=21$ for LL, $n=23$ for LD night, $n=13$ for LD day). Post-hoc comparison for lever presses: $p=0.011$ for LL vs LD night, $p=0.002$ for LL vs LD day. For rewards: $p=0.018$ for LL vs LD night, $p=0.009$ for LL vs LD day. For breaking point: $p=0.011$ for LL vs LD night and $p=0.003$ for LL vs LD day. *$p<0.05$, **$p<0.01$, Games-Howell posttest or Bonferroni's multiple comparison test.

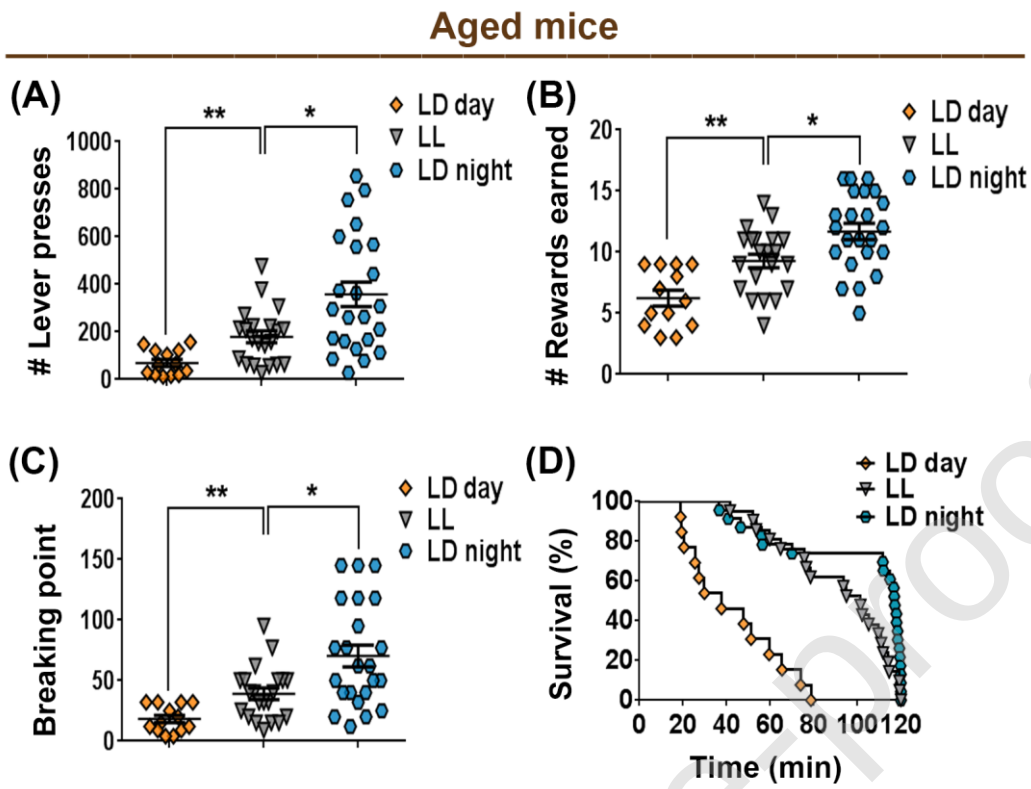

Figure 6. Day/night variation in motivation for food reward without caloric restriction in young mice. Animals under a 12:12 light/dark (LD) cycle were evaluated in the middle of the day (LD day) or in the middle of the night (LD night). Chocolate pellets were used as reward. (A) Total number of lever presses ($p=0.0191$, two-tailed t-test), (B) total number of rewards earned ($p=0.0077$, two-tailed t-test), (C) breaking point ($p=0.0221$, two-tailed t-test), and (D) survival functions for session duration ($p=0.005$, Mantel-Cox test). Data are expressed as mean ± S.E.M (n=20 for LD night, n=15 for LD day). *$p<0.05$, **$p<0.01$, two-tailed t-test.

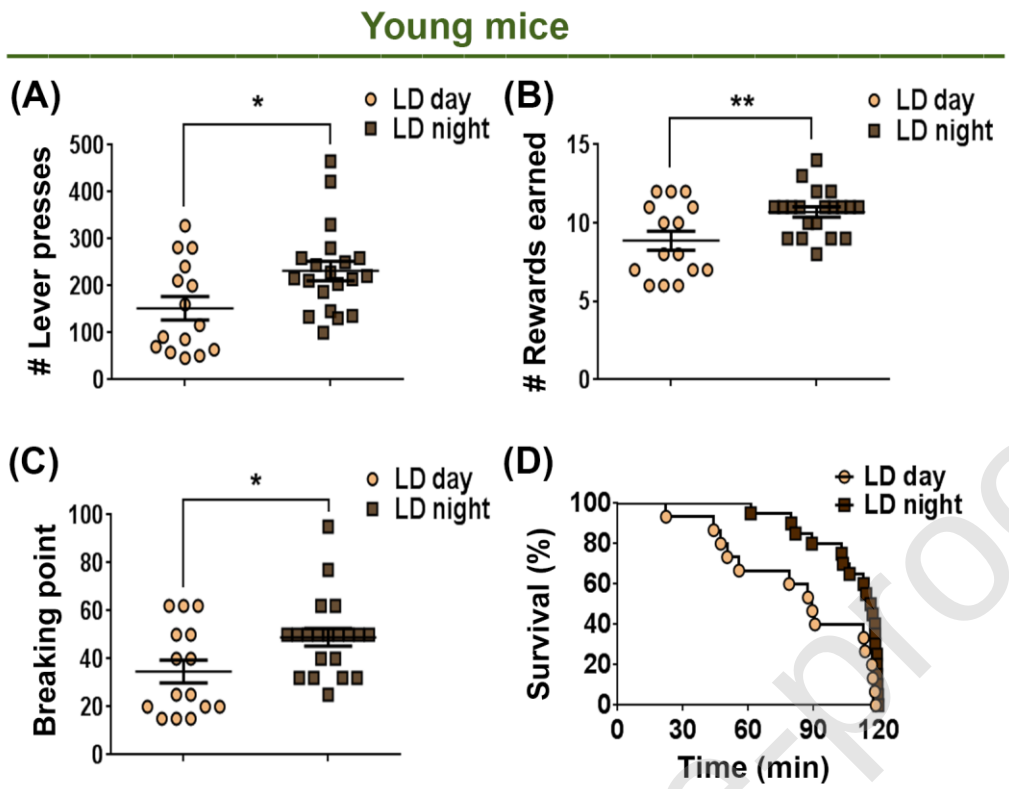

Figure 7. Day/night variation in motivation for food reward without caloric restriction in aged mice. Mice under a 12:12 light/dark (LD) cycle were evaluated in the middle of the day (LD day) or in the middle of the night (LD night). Chocolate pellets were used as reward. (A) Total number of lever presses ($p=0.0004$, two-tailed t-test with Welch's correction), (B) total number of rewards earned ($p<0.0001$, two-tailed t-test), (C) breaking point ($p=0.0003$, two-tailed t-test with Welch's correction), and (D) survival functions for session duration ($p<0.0001$, Mantel-Cox test). Data are expressed as mean ± S.E.M (n=15 for LD night, n=16 for LD day). ***$p<0.001$, two-tailed t-test with or without Welch's correction.